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#### **APPENDIX A**

# Determination of Antimicrobial Activity in a Time Kill Suspension Test

Performed by T.J. Stephens and Associates

Dallas, TX

September, 2000



Thomas J.

## Stephens & Associates Inc.

Clinic
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A Global Research Organization

### DETERMINATION OF THE RAPID GERMICIDAL (TIME KILL) ACTIVITY OF ANTIMICROBIAL PRODUCTS





Stephens & Associates Study Number: L00-D047



In this assay, microbicidal activity is measured by the time kill kinetic method, whereby the survival of organisms exposed to an antimicrobial agent is determined as a function of time. A dilution/aliquot of the test material is brought into contact with a known population of test organisms for a specified period of time, at a specified temperature. At least two contact times are examined; the time points tested in this study were 15, 30, and 60 seconds for test materials 3372-151, and 30 and 60 seconds for test materials. The antimicrobial active is neutralized at the end of the test period. A sample is plated to enumerate the surviving organisms. The percent reduction (in terms of log<sub>10</sub>) from the original population is calculated.

Product performance fell roughly into two groups, A) those which generally demonstrated a 99.9% (3 log<sub>10</sub> reduction) or greater kill efficacy at 60 seconds for most or all bacteria (3372-151, and to a lesser degree, and B) those which generally demonstrated a 90% (1 log<sub>10</sub> reduction) kill efficacy or less at 60 seconds for most or all bacteria (Rhodotorula mucilagiosa and Candida albicans).

Test material 3372-151 demonstrated the best overall kill efficacy. Test material demonstrated a 99.9% or more efficacy for most, but not all bacteria at 30 and 60 seconds. The remaining four test materials were not very effective against most of the microbes tested and were not effective at all against many of them.





#### **PURPOSE**

This time kill study was conducted for Sponsor's test materials as a function of time.

to determine the antimicrobial activity of the

#### **GENERAL INFORMATION**

Stephens & Associates Study Number: L00-D047

Test:

Determination of the Rapid Germicidal (Time Kill) Activity of

**Antimicrobial Products** 

**Test Materials:** 

3372-151

Time Points Examined:

30 and 60 seconds

15, 30, and 60 seconds (3372-151,

Test Organisms:

**Bacteria** 

Burkholderia cepacia ATCC 25416 Citrobacter freundii ATCC 43864 Enterobacter aerogenes ATCC 13048

Enterococcus faecalis MDR (VRE) ATCC 51299

Escherichia coli ATCC 11229

Escherichia coli O157H7 ATCC 43888
Klebsiella pneumoniae ATCC 11296
Pseudomonas aeruginosa ATCC 9027
Salmonella enteritidis ATCC 13076
Salmonella typhimurium ATCC 6539
Serratia marcescens ATCC 14756
Shigella sonnei ATCC 11060

Staphylococcus aureus ATCC 6538

Staphylococcus aureus MRSA ATCC 33592 Streptococcus pneumoniae ATCC 6303 Streptococcus pyogenes ATCC 19615

Yeast

Candida albicans ATCC 10231 Candida tropicalis ATCC 750

Rhodotorula mucilagiosa ATCC 9449

Administrative and Testing Facility:

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Investigator:

J. Stephens Richards, M.S.

Biostatistics Manager:

Heather N.P. Cotsworth, M.S.

Quality Assurance Manager:

Patricia Pierce, M.Ed.



Thomas J. Stephens & Associates, Inc.
Stephens & Associates Study Number: L00-D047

Final Report 11-30-00

GENERAL INFORMATION (Continued)

Sponsor:

Sponsor Representative:

**Experiment Start Date:** 

September 14, 2000

**Experiment Termination Date:** 

October 19, 2000



#### STORAGE, HANDLING, AND DOCUMENTATION OF TEST MATERIALS

The receipt of test materials to Stephens & Associates was documented in a logbook, which serves as a permanent record of the receipt, storage, return, and disposition of all study materials. All study materials were kept in a locked product storage room accessible to laboratory staff members only. At the conclusion of the study, the remaining study materials were returned to the Sponsor.

0148-00L

3372-151

Neat

N/A

Colorless, transparent liquid

#### **TEST MATERIAL DESCRIPTIONS**

Test Material Identification Number (TMIN):

Sponsor Test Material Identification:

Physical Description:

Concentration Tested:

Diluent:

Test Material Identification Number (TMIN):

Sponsor test material identification:

Physical Description:

Concentration Tested:

Diluent:

Test Material Identification Number (TMIN):

Sponsor Test Material Identification:

Physical Description:

Concentration Tested:

Diluent:

Test Material Identification Number (TMIN):

Sponsor Test Material Identification:

Physical Description:

Concentration Tested:

Diluent:

Test Material Identification Number (TMIN):

Sponsor Test Material Identification:

Physical Description:

Concentration Tested:

Diluent:

Test Material Identification Number (TMIN):

Sponsor Test Material Identification:

Physical Description:

Concentration Tested:

Diluent:

#### PROTOCOL AMENDMENTS

Affected Section of Protocol: SPECIAL DIRECTIONS, Contact times

Original Wording:

Revised Wording:

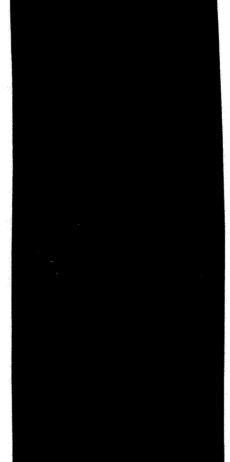
0.5 & 1 minutes

30 and 60 seconds for test materials

15, 30, and 60 seconds for test materials 3372-151,

Reason for Change:

Sponsor request





Thomas J. Stephens & Associates, Inc.

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#### PROTOCOL AMENDMENTS (Continued)

Affected Section of Protocol: Procedure

Original Wording:

Incubate plates for  $48h \pm 2h$  at  $35^{\circ} \pm 2^{\circ}C$ .

Revised Wording:

Incubate plates for 24 to 48 hours at  $35^{\circ} \pm 2^{\circ}$ C.

Reason for Change:

Investigator request

Affected Section of Protocol:

**Procedure** 

Original Wording:

Incubate plates for  $48h \pm 2h$  at  $35^{\circ} \pm 2^{\circ}C$ .

Revised Wording:

Incubate plates containing yeast for 3 to 5 days (at appropriate temperature).

Reason for Change:

Sponsor request

#### PROTOCOL DEVIATION

Affected Section of Protocol:

Procedure

Protocol Specification:

Three trials will be done for each time point tested.

Deviation:

For S. typhimurium, only two trials were completed for the 15 second time

point for test material

Impact of Deviation:

None expected

#### PROCEDURES AND METHODS

Prior to the start of the time kill assay, neutralizer efficacy testing was completed to determine the ability of inactivators to neutralize the killing action of antimicrobial agents found in the test materials. The following microorganisms were used: Candida albicans ATCC 10231, Escherichia coli ATCC 8739, Pseudomonas aeuroginosa ATCC 9027, and Staphylococcus aureus ATCC 6538. TSA+ was used to recover and enumerate bacteria, and SDA+ was used to recover and enumerate yeast. Plates containing bacteria were incubated at 31° to 37°C for 18 to 24 hours, and plates containing yeast were incubated at 30° to 35°C for approximately 48 hours.

#### **Inoculum Preparation**

Bacterial and yeast cultures were seeded and grown, then streaked onto slants. The slants were refrigerated for the duration of the study, and restreaked every seven days. Prior to the use of each organism, cultures from a slant were streaked onto an agar plate and incubated for 24 ± 2 hours. An inoculum was prepared by washing the plate with Phosphate Buffered Saline (PBS), and a suspension prepared from the wash. Each suspension was adjusted spectrophotometrically to a concentration of approximately 108 cfu/ml. The adjusted organism was then serially diluted to approximately 10<sup>3</sup> cfu/ml (the inocula).

#### **Inoculum Enumeration**

One (1.0) ml of each adjusted test organism suspension was serially diluted in PBS to extinction, and 1.0 ml and 0.1 ml aliquots were plated and enumerated to determine the initial level of test organism (cfu/ml).

#### **Neutralizer Toxicity Test**

One (1.0) ml of each adjusted test organism suspension was added to a tube containing D/E neutralizing broth and allowed to stand for approximately 30 minutes. At the end of 30 minutes, 1.0 ml and 0.1 ml aliquots were plated and enumerated. The neutralizer was considered non-toxic up to and including the concentration at which organism recovery was within +/- 50% of the inoculum control counts.



#### PROCEDURES AND METHODS (Continued)

#### **Neutralizer Efficacy Test**

One (1.0) ml of each adjusted test organism suspension was diluted 1:10 in D/E neutralizing broth and 1.0 ml of test material. This mixture was allowed to stand for approximately 30 minutes. At that time, 1.0 ml and 0.1ml aliquots were plated and enumerated. The neutralizer was considered effective if organism recovery was within +/- 50% of the counts for the neutralizer toxicity control.

For the time kill assay, each test material was tested against each of the test organisms (see General Information). For test materials 3372-151, three contact times were examined: 30 and 60 seconds. For test materials 3372-151, three contact times were tested: 15, 30, and 60 seconds. Three trials were done for each time point (plated in duplicate). All procedures were carried out at room temperature. TSA+ was used to recover and enumerate bacteria, and SDA+ was used to recover and enumerate yeast. Bacterial plates were incubated at 35° ± 2°C for 24 to 48 hours, and yeast plates were incubated at 20° to 25° C for three to five days. TSA II with 5% sheep blood was used to recover *S. pneumoniae*; these plates were incubated at 37°C and 4% CO<sub>2</sub> for approximately 48 hours.

#### **Inoculum Preparation and Enumeration**

Cultures were grown and prepared as described for neutralizer efficacy testing. Each organism suspension was adjusted spectrophotometrically to a concentration of approximately 10<sup>8</sup> cfu/ml in PBS. Prior to the start of testing each day, a "pre-test" count was done: the adjusted test organism suspension was serially diluted in Butterfield's Phosphate Buffer plus Lecithin and Tween 80 (BPB+) to 10<sup>2</sup> cfu/ml, and 0.1 and 1.0 ml aliquots were plated (dilutions 10<sup>-8</sup> and 10<sup>-7</sup>) and enumerated.

#### Blank

A beaker containing 100 grams of sterile deionized water was inoculated with 1.0 ml of adjusted test organism suspension (approximately 10<sup>8</sup> cfu/ml). At the longest time point (60 seconds), two serial dilutions were made (1:100) in D/E neutralizing broth, and 0.1 and 1.0 ml aliquots were plated (dilutions 10<sup>-4</sup> and 10<sup>-5</sup>) and enumerated.

#### **Time Kill Assay**

One hundred (100) grams of each test material was placed in a beaker and tempered with constant stirring. Test material 3372-151 was tested neat, and test materials and were tested at 75% concentration. The sample was inoculated with 1.0 ml of adjusted test organism suspension (approximately 10<sup>8</sup> cfu/ml). At 30 and 60 seconds, or at 15, 30, and 60 seconds, depending on the test material being examined, two serial dilutions were made of the test material/organism mixture in D/E neutralizer broth (1:100 and 1:10). One tenth (0.1) ml and 1.0 ml aliquots were plated (dilutions  $10^{-2}$ ,  $10^{-3}$ , and  $10^{-4}$ ) and enumerated.

#### CALCULATIONS AND DATA MANAGEMENT

Determination of reduction was calculated by averaging plate counts (cfu/g), correcting for dilution, and converting to  $\log_{10}$ . For each time point, the  $\log_{10}$  for the three trials was averaged to give a mean  $\log_{10}$ . Counts after exposure to test material were compared to the blank to determine  $\log_{10}$  reduction using the following formula:  $\log_{10}$  reduction =  $\log_{10}$  blank - mean  $\log_{10}$  sample. Results were reported as the mean  $\log_{10}$  reduction of the three trials at each time point for each test organism.

#### MAINTENANCE OF RECORDS

All original records (including the study protocol, data sheets, and any other records or forms used in this study) and a copy of the final report will be retained on file in the Stephens & Associates archives for two years from the date of study completion. When the archive time has expired, the study files will either be sent to the Sponsor or destroyed. Any of these options may be carried out upon the Sponsor's request and notification.



#### **RESULTS**

One hundred (100) grams of each test material was inoculated with 1.0 ml of each adjusted test organism suspension. At 30 and 60 seconds (3372-151, the active agent was neutralized, and samples were plated to enumerate the surviving organisms. Average plate counts were converted to  $\log_{10}$ , and the mean  $\log_{10}$  of each trial was compared to the blank to determine the  $\log_{10}$  reduction. Table 1 presents the mean  $\log_{10}$  reductions of each trial for each time point and test organism.

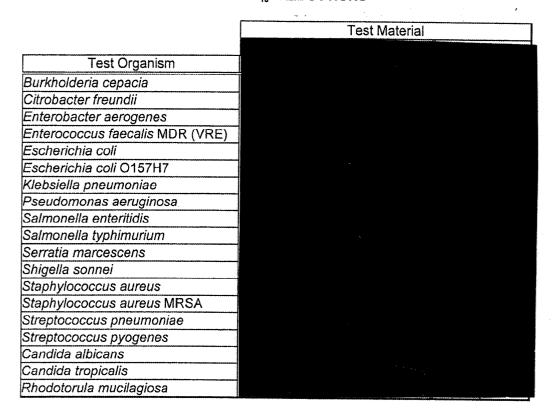
TABLE 1
MEAN LOG<sub>10</sub> REDUCTIONS

		***************************************	<del></del>	Test Material
		3372-15 <sup>-</sup>	1	
Test Organism	15 sec	30 sec	60sec	
Burkholderia cepacia	>4.02	>4.02	>4.02	
Citrobacter freundii	2.00	4.12	>4.99	
Enterobacter aerogenes	1.72	2.69	>5.10	
Enterococcus faecalis MDR (VRE)	4.30	4.34	>4.79	
Escherichia coli	>4.26	>4.26	>4.26	
Escherichia coli O157H7	1.25	2.88	>4.87	
Klebsiella pneumoniae	>3.99	>3.99	>3.99	
Pseudomonas aeruginosa	>3.43	>3.43	>3.43	
Salmonella enteritidis	4.36	>5.04	>5.04	
Salmonella typhimurium	2.93	>4.36	>4.36	
Serratia marcescens	0.63	1.21	4.05	
Shigella sonnei	0.69	2.10	3.15	
Staphylococcus aureus	>4.21	>4.21	>4.21	
Staphylococcus aureus MRSA	2.61	2.89	2.53	
Streptococcus pneumoniae	>4.03	>4.03	>4.03	
Streptococcus pyogenes	>4.54	>4.54	>4.54	
Candida albicans	2.49	2.23	2.64	
Candida tropicalis	0.46	0.68	0.72	
Rhodotorula mucilagiosa	1.51	2.91	3.25	



#### **RESULTS (Continued)**

### TABLE 1 (Continued) MEAN LOG<sub>10</sub> REDUCTIONS



#### **DISCUSSION AND CONCLUSIONS**

Table 1 provides a concise summary of the six products assayed. Product performance fell roughly into two groups, A) those which generally demonstrated a 99.9% (3 log<sub>10</sub> reduction) or greater kill efficacy at 60 seconds for most or all bacteria (3372-151, and to a lesser degree, but those which generally demonstrated a 90% (1 log<sub>10</sub> reduction) kill efficacy or less at 60 seconds for most or all bacteria Only one product (3372-151) demonstrated efficacy against yeasts (99.9% kill at 60 seconds for *Rhodotorula mucilagiosa* and 99.5% for *Candida albicans*; it was not effective against *Candida tropicalis*). The other five products did not show significant efficacy against yeasts.

Test material 3372-151 demonstrated the best overall kill efficacy. Test material a 99.9% or more efficacy for most, but not all bacteria at 30 seconds and at 60 seconds. The remaining four test materials were not very effective against most of the microbes tested and were not effective at all against many of them. Specific performance is summarized in Table 1 and the Time Kill Summary Chart (Appendix I).





#### STATEMENT OF QUALITY ASSURANCE

All data and supporting documentation for this study have been audited by the Stephens & Associates, Inc., Quality Assurance Department and found to be accurate, complete, and in compliance with the requirements of the protocol and Stephens & Associates' Standard Operating Procedures. This report has been reviewed and accurately reflects all aspects of the conduct of the study.

All laboratory research studies that are performed by Stephens & Associates are in accordance with federal regulations and Good Laboratory Practice guidelines.

Patricia Pierce, M.Ed.

Quality Assurance Manager

<u>ව/ග</u> Date



#### **REPORT APPROVAL**

Report approved by:

THOMAS J. STEPHENS & ASSOCIATES, INC.

J. Stephen Richards, Investigator

#### **APPENDICES**

- I. Biostatistics
- II. Graphs
- III. Protocol: Determination of the Rapid Germicidal (Time Kill) Activity of Antimicrobial Products
- IV. Copies of Data Sheets





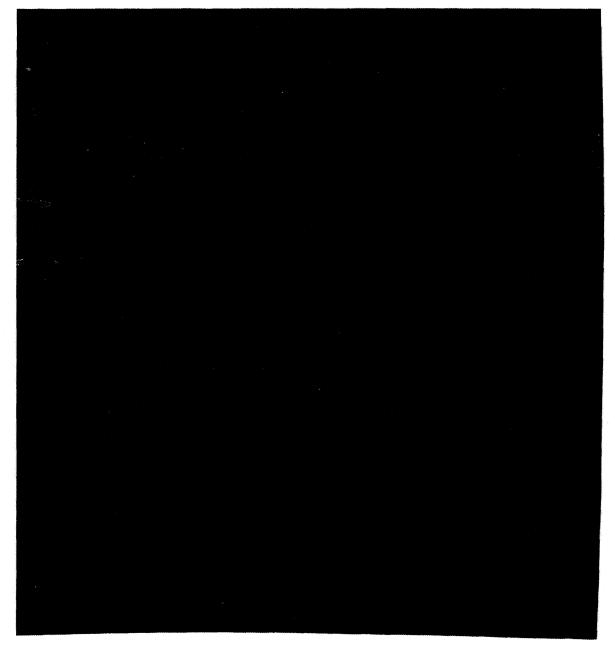
#### I. BIOSTATISTICS

TIME LL SUMMARY

Product Code		3372-151		
	LOC	REDUC	TION	
	15 sec	30 sec	1 min	
		<del> </del>		
Staphylococcus aureus	>4.21	>4.21	>4.21	
Staphylococcus aureus				
MRSA	2.61	2.89	2.53	
	<del> </del>			
Escherichia coli	>4.26	>4.26	>4.26	
Egenerican con				
Escherichia coli O157H7	1.25	2.88	>4.87	
Enterococcus faecalis MDR				
	4.30	4.34	>4.79	
(VRE)				
<b>C</b> :	>4.54	>4.54	>4.54	
Streptococcus pyogenes				
	>4.03	>4.03	>4.03	
Streptococcus pneumoniae			,,,,,	
	1.72	2.69	>5.10	
Enterobacter aerogenes			5.10	
	>3.99	>3.99	>3.99	
Klebsiella pneumoniae	3.77	3.77	3.77	
	0.63	1.21	4.05	
Serratia marcescens	0.05	1.21	1.03	
	4.36	>5.04	>5.04	
Salmonella enteritidis	1.50	2.01	73.04	
	2.93	>4.36	>4.36	
Salmonella typhimurium	2.75	50	74.50	
	0.69	2.10	3.15	
Shigella sonnei	0.07	2.10	3.13	
	2.00	4.12	>4.99	
Citrobacter freundii	2.00	7.12	74.33	
	>3.43	>3.43	>3.43	
Pseudomonas aeruginosa	~3. <del>4</del> 3	~3.43	73.43	
	>4.00	>4.00	> 4 00	
Burkholderia cepacia	>4.02	>4.02	>4.02	
	0.40	2.22	2 (1	
Candida albicans	2.49	2.23	2.64	
	A 4.4	A 20	0.50	
Candida tropicalis	0.46	0.68	0.72	
Rhodotorula mucilagiosa	1.51	2.91	3.25	
		L	<u> </u>	

S. aureus 6538

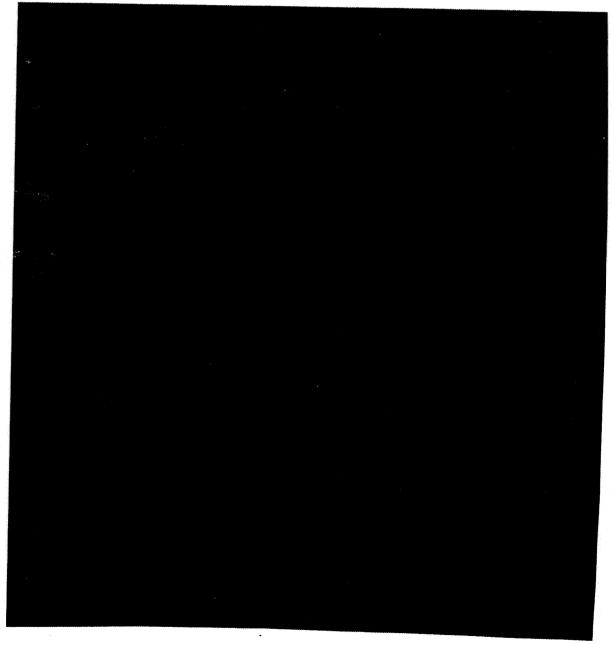
Test Material	Time	Trial #	Log of the Trial	Avg of Trials	Log of the Blank	Log Reduction
3372-151	15 seconds	1	2.00	2.00	6.21	4.21
		2	2.00			
		3	2.00			
	30 seconds	1	2.00	2.00	6.21	4.21
		2	2.00			
		3	2.00			
(	60 seconds	1	2.00	2.00	6.21	4.21
		2	2.00			
		3	2.00			





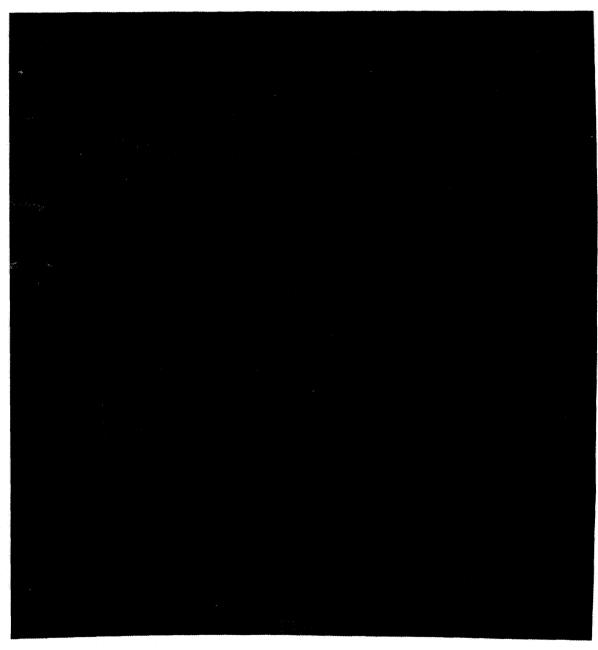
S. aureus 33592

Test Material	Time	Trial #	Log of the Trial	Avg of Trials	Log of the Blank	Log Reduction
	15 seconds	1	4.33	4.09	6.70	2.61
		2	3.81			
		3	4.13			
	30 seconds	1	3.61	3.81	6.70	2.89
		2	3.89			
		3	3.93			
	60 seconds	1	4.34	4.17	6.70	2.53
		2	4.08			
		3	4.08			



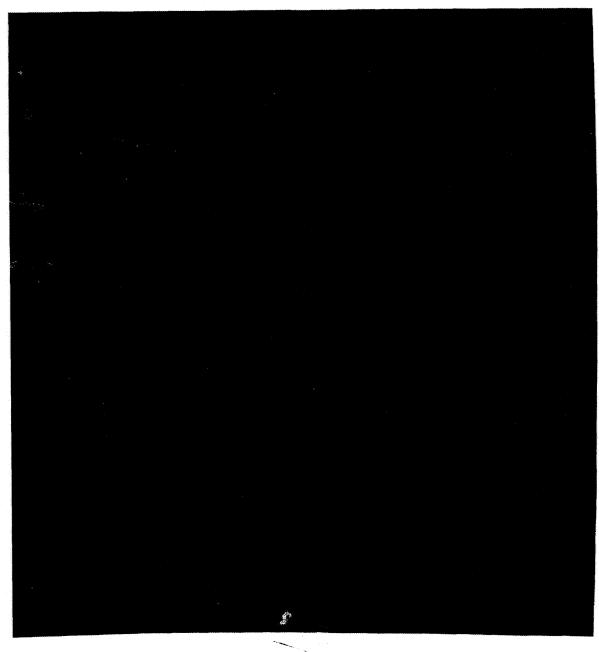
E. coli 11229

Test Material	Time	Trial#	Log of the Trial	Avg of Trials	Log of the Blank	Log Reduction
3372-151	15 seconds	1	2.00	2.00	6.26	4.26
		2	2.00			
		3	2.00			
	30 seconds	1	2.00	2.00	6.26	4.26
		2	2.00			
		3	2.00			
	60 seconds	1	2.00	2.00	6.26	4.26
		2	2.00			
		3	2.00			



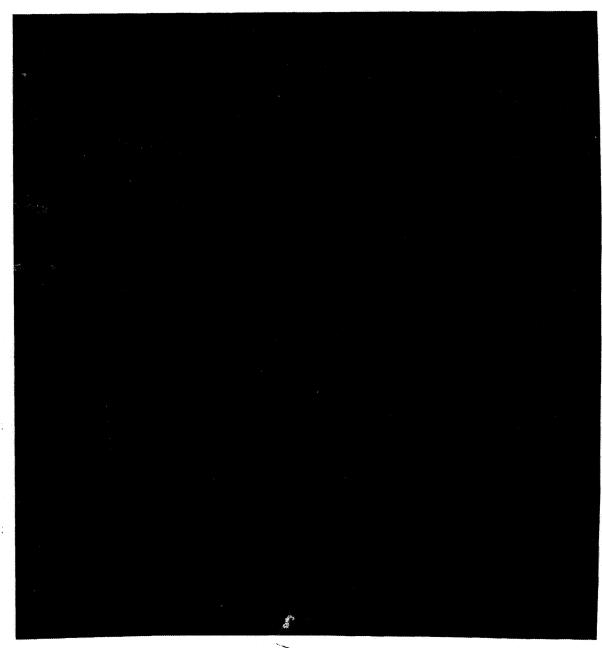
E. coli 43888

Test Material	Time	Trial#	Log of the Trial	Avg of Trials	Log of the Blank	Log Reduction
3372-151	15 seconds	1	5.13	5.62	6.87	1.25
		2	5.82			
		3	5.90			
	30 seconds	1	3.84	3.99	6.87	2.88
		2	4.02			
		3	4.11			
60 s	60 seconds	1	2.00	2.00	6.87	4.87
		2	2.00			
		3	2.00			



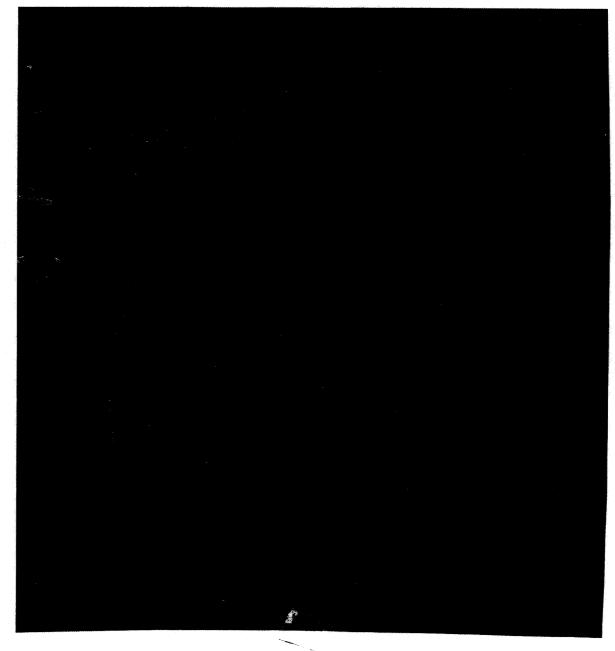
E. faecalis 51299

Test Material	Time	Trial #	Log of the Trial	Avg of Trials	Log of the Blank	Log Reduction
3372-151 15	15 seconds	1	3.47	2.49	6.79	4.30
		2	2.00			
		3	2.00			
	30 seconds	1	2.00	2.45	6.79	4.34
		2	2.18			
		3	3.18			
60 seconds	60 seconds	1	2.00	2.00	6.79	4.79
		2	2.00			
		3	2.00			



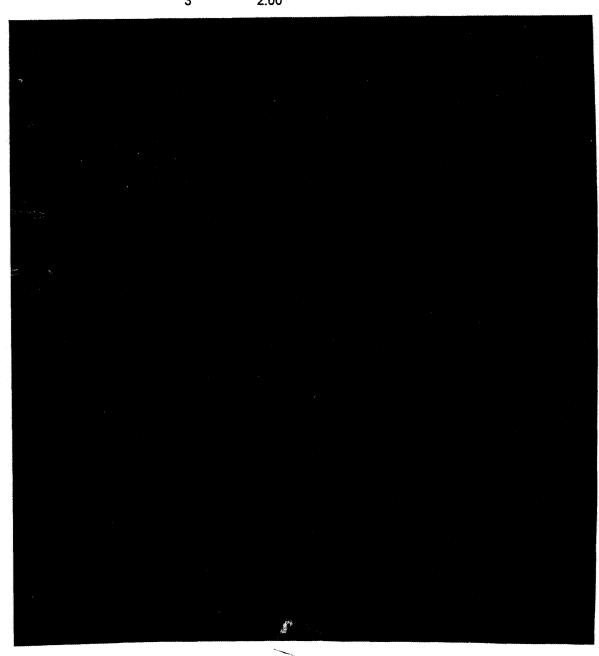
S. pyogenes 19615

Test Material	Time	Trial#	Log of the Trial	Avg of Trials	Log of the Blank	Log Reduction
3372-151	15 seconds	1	2.00	2.00	6.54	4.54
		2	2.00			
		3	2.00			
3	30 seconds	1	2.00	2.00	6.54	4.54
		2	2.00			
		3	2.00			
60 s	60 seconds	1	2.00	2.00	6.54	4.54
		2	2.00			
		3	2.00			



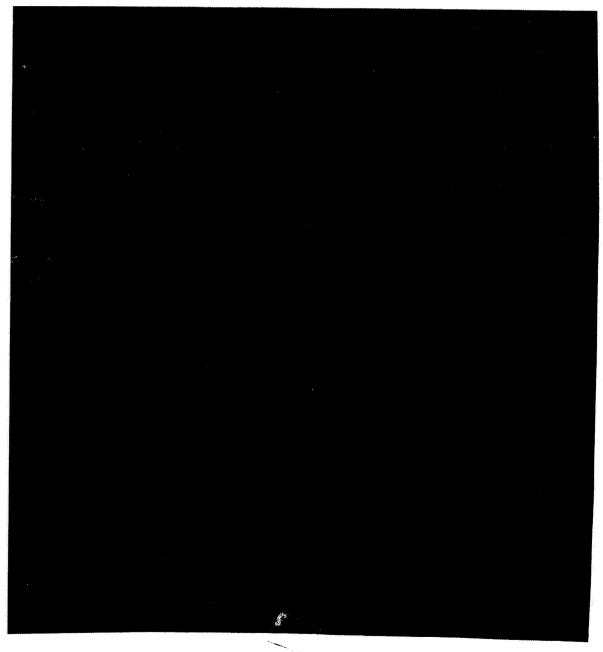
S. pneumoniae 6303

Test Material	Time	Trial#	Log of the Trial	Avg of Trials	Log of the Blank	Log Reduction
3372-151 15 seco	15 seconds	1	2.00	2.00	6.03	4.03
		2	2.00			
		3	2.00			
	30 seconds	1	2.00	2.00	6.03	4.03
		2	2.00			
		3	2.00			
60 seco	60 seconds	1	2.00	2.00	6.03	4.03
		2	2.00			
		3	2.00			



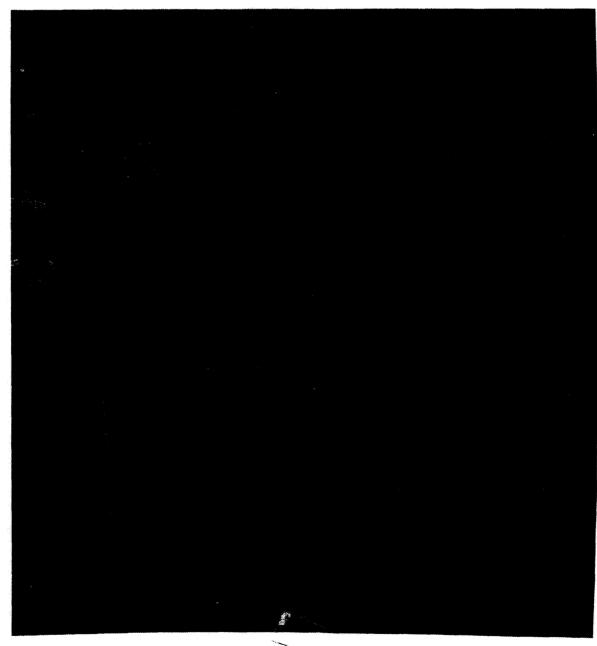
E. aerogenes 13048

Test Material	Time	Trial #	Log of the Trial	Avg of Trials	Log of the Blank	Log Reduction
3372-151	15 seconds	1	5.35	5.38	7.10	1.72
30 sec		2	5.43			*******
		3	5.35			
	30 seconds	1	4.17	4.41	7.10	2.69
		2	4.46			
		3	4.60			
60	60 seconds	1	2.00	2.00	7.10	5.10
		2	2.00			
		3	2.00			



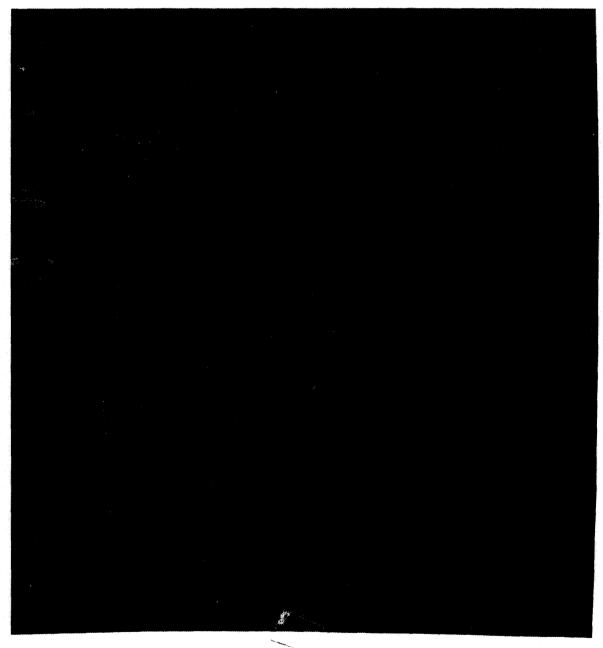
#### K. pneumoniae 11296

Test Material	Time	Trial#	Log of the Trial	Avg of Trials	Log of the Blank	Log Reduction
3372-151	15 seconds	1	2.00	2.00	5.99	3.99
		2	2.00			
		3	2.00			
	30 seconds	1	2.00	2.00	5.99	3.99
		2	2.00			
		3	2.00			
	60 seconds	1	2.00	2.00	5.99	3.99
		2	2.00			
		3	2.00			



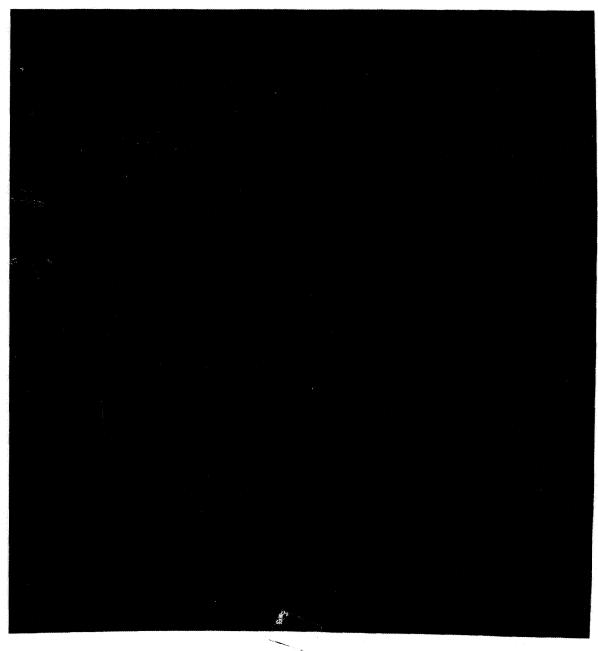
S. marcescens 14756

Test Material	Time	Trial #	Log of the Trial	Avg of Trials	Log of the Blank	Log Reduction
3372-151	15 seconds	1	5.67	5.77	6.40	0.63
		2	6.08			
		3	5.57			
	30 seconds	1	5.15	5.19	6.40	1.21
		2	5.32			
		3	5.11			
6	60 seconds	1	2.40	2.35	6.40	4.05
		2	2.18			
		3	2.48			



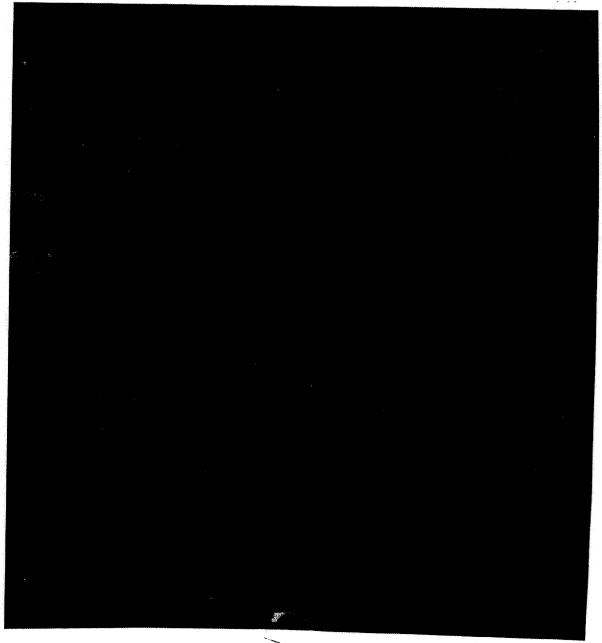
S. enteritidis 13076

Test Material	Time	Trial #	Log of the Trial	Avg of Trials	Log of the Blank	Log Reduction
3372-151	15 seconds	1	2.70	2.68	7.04	4.36
		2	2.18			
		3	3.15			
	30 seconds	1	2.00	2.00	7.04	5.04
		2	2.00			
		3	2.00			
	60 seconds	1	2.00	2.00	7.04	5.04
		2	2.00			
		3	2.00			



S. typhimurium 6539

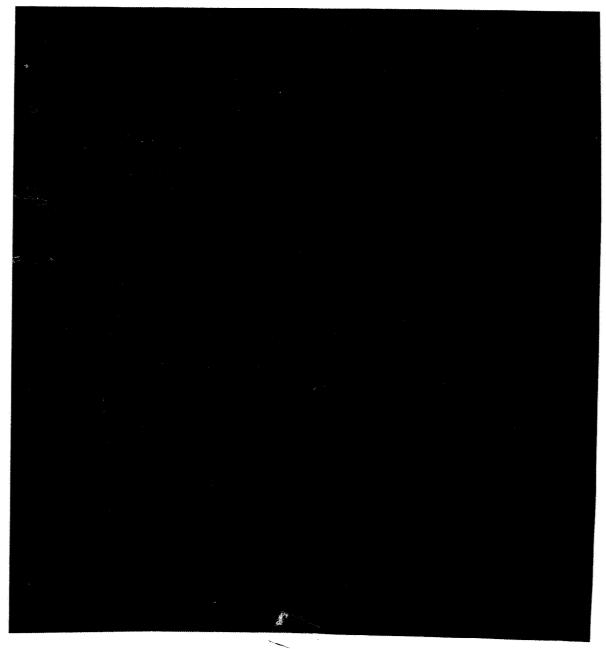
Test Material	Time	Trial #	Log of the Trial	Avg of Trials	Log of the Blank	Log Reduction
3372-151	15 seconds	1	3.81	3.43	6.36	2.93
		2	2.74			
		3	3.74			
	30 seconds	1	2.00	2.00	6.36	4.36
		2	2.00			
		3	2.00			
	60 seconds	1	2.00	2.00	6.36	4.36
		2	2.00			
		3	2.00			





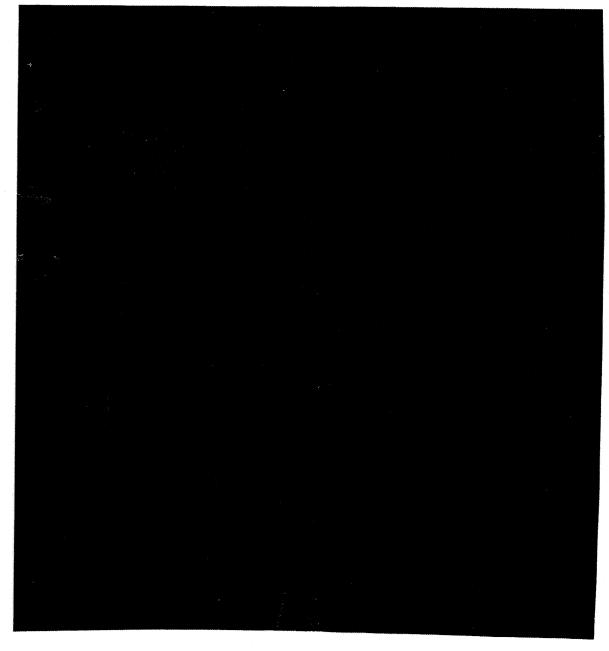
S. sonnei 11060

Test Material	Time	Trial #	Log of the Trial	Avg of Trials	Log of the Blank	Log Reduction
3372-151	15 seconds	1	5.29	5.25	5.94	0.69
		2	5.16			
		3	5.30			
	30 seconds	1	3.66	3.84	5.94	2.10
		2	3.83			
		3	4.03			
	60 seconds	1	2.90	2.79	5.94	3.15
		2	2.81			
		3	2.65			



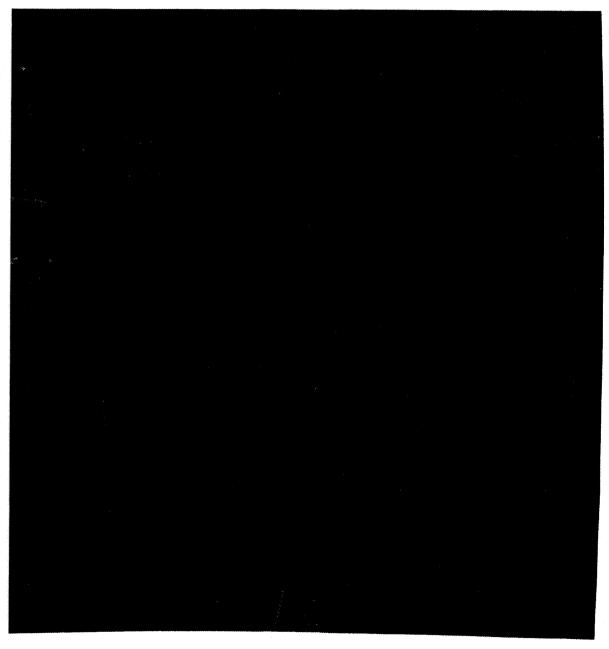
C. freundii 43864

Test Material	Time	Trial#		Avg of Trials	Log of the Blank	Log Reduction
3372-151	15 seconds	1	5.08	4.99	6.99	2.00
		2	4.89			~
		3	4.99			
	30 seconds	1	3.20	2.87	6.99	4.12
		2	2.88			
		3	2.54			
	60 seconds	1	2.00	2.00	6.99	4.99
		2	2.00			
		3	2.00			



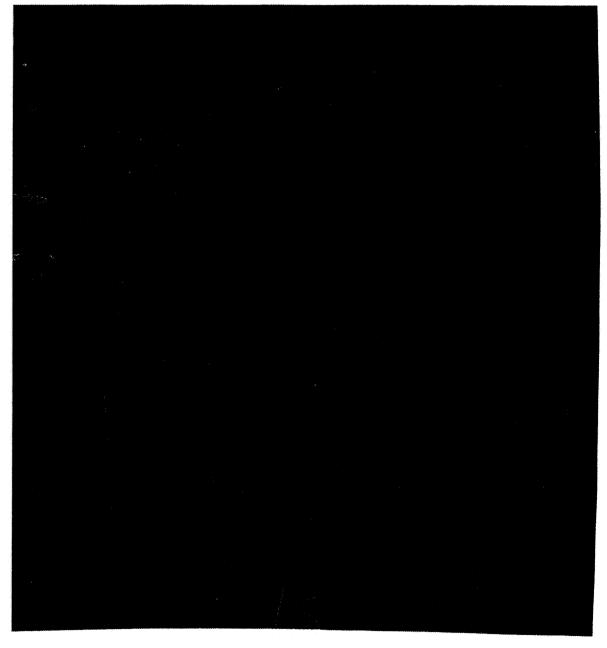
P. aeruginosa 9027

Test Material	Time	Trial #	Log of the Trial	Avg of Trials	Log of the Blank	Log Reduction
3372-151	15 seconds	1	2.00	2.00	5.43	3.43
		2	2.00			0.40
		3	2.00			
	30 seconds	1	2.00	2.00	5.43	3.43
		2	2.00			00
		3	2.00			
	60 seconds	1	2.00	2.00	5.43	3.43
		2	2.00			31.0
		3	2.00			



B. cepacia 25416

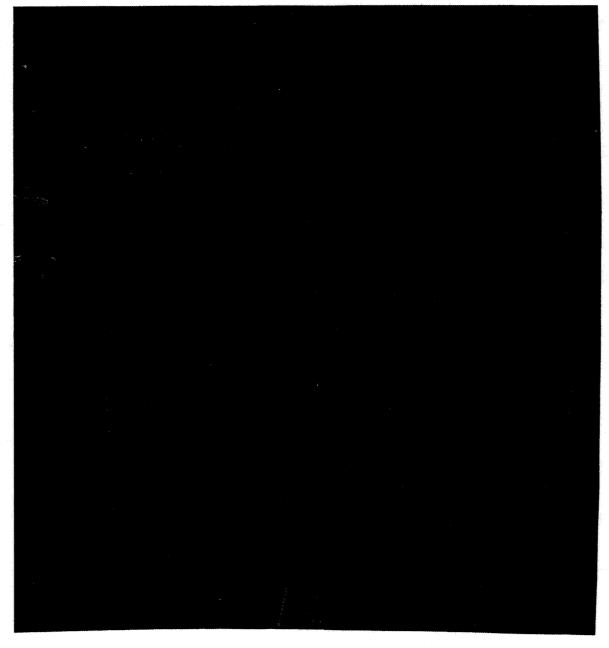
Test Material	Time	Trial #	Log of the Trial	Avg of Trials	Log of the Blank	Log Reduction
3372-151	15 seconds	1	2.00	2.00	6.02	4.02
		2	2.00			1.02
		3	2.00			
	30 seconds	1	2.00	2.00	6.02	4.02
		2	2.00			
		3	2.00			
	60 seconds	1	2.00	2.00	6.02	4.02
		2	2.00			
		3	2.00			
	60 seconds	3 1 2	2.00 2.00 2.00	2.00		4.02





C. albicans 10231

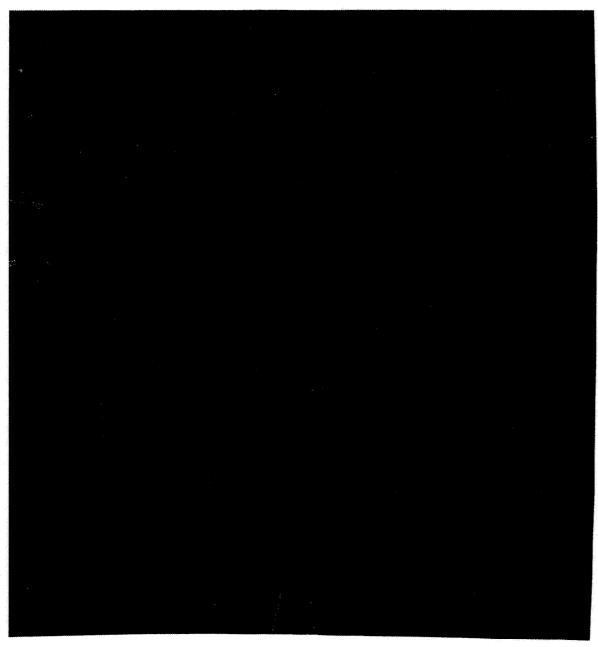
Time	Trial #	Log of the Trial		Log of the Blank	Log Reduction
15 seconds	1	3.64	3.72	6.21	2.49
	2	2.88			<b></b> , 0
	3	4.65			
30 seconds	1	4.20	3.98	6.21	2.23
	2	3.95			
	3	3.78			
60 seconds	1	3.04	3.57	6.21	2.64
	2	4.52			
	3	3.16			
	15 seconds 30 seconds	15 seconds 1 2 3 3 30 seconds 1 2 3 60 seconds 1 2	15 seconds 1 3.64 2 2.88 3 4.65 30 seconds 1 4.20 2 3.95 3 3.78 60 seconds 1 3.04 2 4.52	15 seconds 1 3.64 3.72 2 2.88 3 4.65 30 seconds 1 4.20 3.98 2 3.95 3 3.78 60 seconds 1 3.04 3.57 2 4.52	15 seconds 1 3.64 3.72 6.21 2 2.88 3 4.65 30 seconds 1 4.20 3.98 6.21 2 3.95 3 3.78 60 seconds 1 3.04 3.57 6.21 2 4.52





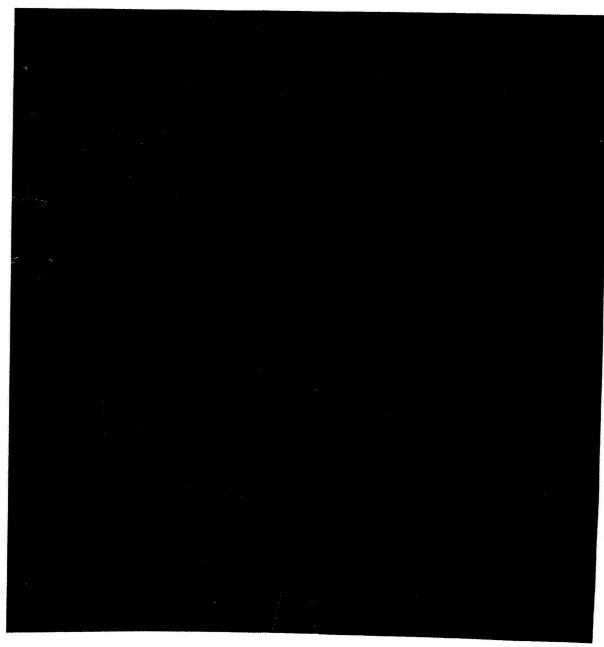
C. tropicalis 750

Test Material	Time	Trial #	Log of the Trial	Avg of Trials	Log of the Blank	Log Reduction
3372-151	15 seconds	1	4.37	4.34	4.80	0.46
		2	4.32			
		3	4.32			
	30 seconds	1	4.02	4.12	4.80	0.68
		2	4.03			
		3	4.30			
	60 seconds	1	4.11	4.08	4.80	0.72
		2	4.04			
•		3	4.08			



R. mucilagiosa 9449

Test Material	Time	Trial#	Log of the Trial	Avg of Trials	Log of the Blank	Log Reduction
3372-151	15 seconds	1	4.69	4.55	6.06	1.51
		2	4.38		0.50	1.01
		3	4.57			
	30 seconds	1	3.41	3.15	6.06	2.91
		2	3.02			2.01
		3	3.02			
	60 seconds	1	3.58	2.81	6.06	3.25
		2	2.00			0.20
		3	2.84			



III. PROTOCOL



STUDY NUMBER: L00-D047

TEST MATERIAL IDENTIFICATION: ATTACHMENT 2

SPONSOR:

SPONSOR'S REPRESENTATIVE:

**TESTING FACILITY:** 

Thomas J. Stephens & Associates, Inc. 3310 Keller Springs Road, Suite 130

Carrollton, TX 75006 (972) 392-1529 (972) 392-2347 (fax)

INVESTIGATOR: J. Stephen Richards, M.S.

Director, Laboratory Sciences

Thomas J. Stephens & Associates, Inc.

PROPOSED STARTING DATE: Week of September 11, 2000

PROPOSED COMPLETION DATE: Week of October 2, 2000

APPROVALS: I have reviewed and approve the protocol to be used in

this study.

Investigators J. Stephen Ric

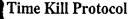
Sponsor:

10/19/2000 Date

Confidential

Title: Time Kill

Effective Date: August 31, 2000



# **PURPOSE**

The purpose of this study is to determine the antimicrobial activity of antimicrobial agents as a function of time.

# **JUSTIFICATION**

The Kill Test provides an *in vitro* method for demonstrating that an antimicrobial active retains activity after incorporation in a product formulation.

# **ASSAY PROCEDURE**

Prior to initiating the time kill test, neutralization needs will be assessed and neutralizer selection and efficacy determined (See Attachment III). The time kill testing will be performed as specified and directed in Research Test Method No.: See Attachment I:

Document; Research Test Method No.:

"Determination of the Rapid Germicidal (Time Kill) Activity of Antimicrobial Products"

# SPECIAL DIRECTIONS (As specified by

Contact times: 0.5 & 1 minutes

Organisms:

Staphylococcus aureus	6538
Staphylococcus aureus MRSA	33592
Escherichia coli	11229
Escherichia coli O157H7	43888
Enterococcus faecalis MDR (VRE)	51299
Streptococcus pyogenes	19615
Streptococcus pneumoniae	6303
Enterobacter aerogenes	13048
Klebsiella pneumoniae	11296
Serratia marcescens	14756
Salmonella enteritidis	13076
Salmonella typhimurium	6539
Shigella sonnei	11060
Citrobacter freundii	43864
Pseudomonas aeruginosa	9027
Burkholderia cepacia	25416
Candida albicans	10231
Candida tropicalis	750
Rhodotorula mucilagiosa	9449

# **Test Concentration:**

Identification Number	Test Concentration
3372-151	100%
	75%
- Harris Garage	75%
	75%
	75%
	75%

Testing to be performed in triplicate with all appropriate controls.



Thomas J. Stephens & Associates, Inc.

#### Confidential

Title: Time Kill Effective Date: August 31, 2000

# REGULATORY AGENCY SUBMISSION

This study will be subject to regulatory agency (FDA) review.

# PROTOCOL AMENDMENTS/DEVIATIONS

If the study design for an approved protocol needs to be revised, a protocol amendment or deviation (whichever is applicable) will be written describing the change. The amendment or deviation will be signed by the Study Investigator and sent to the Sponsor for an approval signature. Changes or clarifications to approved protocols that do not affect study conduct/design, or final results (for example: typos, administrative changes, proposed start/end dates) will be documented in a file memo.

# **CALCULATIONS/DATA ANALYSIS**

See Attachment I

# GOOD LABORATORY PRACTICE REGULATIONS

This protocol will be conducted according to the principles of Good Laboratory Practice regulations. If it becomes necessary to change this approved protocol, verbal agreement to make such a change will be made between the study director and the Sponsor.

As soon as practical, the change and reasons for it will be written and signed by the study director and Sponsor. This document will then be attached to the protocol as an addendum. Examples of significant changes requiring Sponsor's approval are listed below:

- -Changes in the test materials to be tested
- -Changes in the concentrations of the test materials.
- -Changes in the assay procedure.

# **FINAL REPORT**

A final report will be issued to the Sponsor which will include the following information:

- -Descriptions of the test material(s).
- -Dates of study initiation and completion
- -Any protocol deviation(s).
- -Study results, statistical calculations, and a summary/conclusion
- -A copy of this protocol
- -Copy of media QC records / Temperature charts / Culture Maintenance records
- -Copy of Raw Data

#### **RETENTION OF TEST MATERIALS**

At the completion of the study, remaining test materials will, at the Sponsor's expense, be returned to the Sponsor or destroyed at the Sponsor's expense.

### NOTIFICATION OF CHEMICAL HAZARDS

When sending test materials that are chemical hazards, the Sponsor must also supply appropriate MSDS information. Testing will not be done without this information. If hazardous materials are supplied, the materials will be returned to the Sponsor at the Sponsor's expense after completion of the study.

# MAINTENANCE OF RECORDS

When the final report is completed, all original records (including the study protocol, raw data, and any other records or forms used in the study) and a copy of the final report will be retained on file in the Stephens & Associates' archives for two years. When the archive time has expired, the study files are to either be sent to the Sponsor, archived at a different location at the Sponsor's expense, or destroyed. Any of these options may be carried out upon the Sponsor's request and notification.

# **REFERENCES:**

See Attachment I

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Title: Time Kill
Effective Date: Date signed by
Sponsor

# ATTACHMENT I



"Determination of the Rapid Germicidal (Time Kill) Activity of Antimicrobial Products"



#### General:

Microbicidal activity is measured by the time-kill kinetic method, whereby the survival of organisms exposed to an antimicrobial agent is determined as a function of time. A dilution/aliquot of the test material is brought into contact with a known population of test organisms for a specified period of time, at a specified temperature. The antimicrobial active is neutralized at the end of the test period. A sample is plated to enumerate the surviving organisms. The percent reduction from the original population is calculated.

### Discussion:

# SAMPLE PREPARATION

Sample concentrations are 0-50% for liquids and 0-10% for solids. Test temperature will be determined by investigator. Note: Bar soap solutions greater than 5% w/v begin to gel at temperatures below 30° C. All testing is performed in triplicate (3 replicates) - each replicate is plated in duplicate with TSA+. Thoroughly disperse test product in sterile DI water. QS samples to 100 g in sterile 250 mL beakers containing magnetic stir bars.

# CONTACT TIME

At least two contact times are examined. The first time point should be between 1 and 30 seconds. Other times may be tested at the discretion of the investigator.

# CONTACT TEMPERATURE

Temperature may vary dependent upon test product. A temperature of 250 +/- 20 C is recommended for liquids and 37° +/- 2° C for solids.

Originator	/Date	Department	Manager/Date	Additional Dep (if applic	artment's Manager cable)/Date Page 1
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Approved By:					
***************************************					
hange(s): New					



### Inoculum:

- 1. Grow bacterial cultures according to SOP \_\_\_\_\_\_, Section 5.5 through 5.7.
- 2. Prepare decimal dilutions in BPB+ to 10<sup>-6</sup>.
- 3. Plate 10<sup>-6</sup> and 10<sup>-7</sup> aliquots in triplicate.
- 4. Pour 15 20 mL of sterile molten TSA+ into petri plates for bacteria. Mix by rotating plates to disperse inoculum in the agar completely. Stack series of plates on trays and allow agar to solidify.
- 5. If the test period is longer than 60 minutes repeat steps 2-4 and identify each series of plates as either "pre" or "post" count.
- 6. The beginning and end counts must be within one log<sub>10</sub> for test to be valid.
- 7. Incubate plates on trays at 35° +/- 2° C for 48 h +/- 2 h. Count colonies with aid of a Quebec colony counter and record as cfu/mL.

# Test Organism(s):

Other organisms may be used at the discretion of the investigator.

Gram Positive Organisms	Gram Negative Organisms
Staphylococcus aureus ATCC 6538	Escherichia coli ATCC 11229
Staphylococcus aureus (mrsa) ATCC 33592	Klebsiella pneumoniae ATCC 10031
Staphylococcus epidermidis ATCC 12228	Pseudomonas aeruginosa ATCC 9027
Streptococcus pyogenes ATCC 19615	Serratia marcescens ATCC 14756
Streptococcus pneumoniae ATCC 6303	Salmonella choleraesuis ATCC 10708
Enterococcus faecalis (vre) ATCC 51299	
Corynebacterium minutissimum ATCC 23348	

#### Materials: all to be sterilized before use

Tryptic Soy Broth (TSB).

Trypticase Soy Agar with Lecithin and Polysorbate 80 (TSA+).

Neutralizer - Select D/E neutralizing Broth or equivalent neutralizing agent appropriate for active ingredient. If neutralizer other than D/E broth is used, a neutralizer effectiveness test must be performed. 9.0 mL and 9.9 mL D/E broth (D/E Neutralizing Broth) blanks.

DI water.

Physiological saline.

100 uL and 1000 uL pipette tips.

9.0 mL BPB+ (Butterfield's Phosphate Buffer plus Lecithin and Tween 80) blanks.

Four mm. transfer loops.

250 mL sterile glass beakers with magnetic stirring rods.

### Equipment:

Submersible rotor powered magnetic stirrers or equivalent.

Waterbaths with temperature controller - one for dissolving samples, one for performing test.

Quebec colony counter.

Incubator capable of maintaining 350 +/- 20 C.

Colony counter - hand held tally.

Pippetors capable of handling tips mentioned above.

# CONFIDENTIAL

# Research Test Method No.:



controlled document if the logo is in color

Document

Revision No.:

#### Procedure:

- 1. Place beaker in water bath until test material is in solution. Insure that sample is completely dissolved before proceeding. For some solid samples the water bath temperature may need to be up to 42° C.
- 2. Temper sample at test temperature with constant stirring (37° +/- 2° C for solids, 25° +/- 2° C for liquids).
- 3. Inoculate sample with 1.0 mL of test organism (approximately 10<sup>8</sup> CFU/mL).
- 4. At designated time(s), transfer 0.1 mL of sample/bacteria mixture from beaker into a 9.9 mL D/E broth blank (10<sup>-2</sup> dilution). This is a 1:100 dilution.
- 5. Transfer 1.0 mL of this mixture into a 9.0 mL D/E broth blank (10<sup>-3</sup> dilution). This is a 1:10 dilution from the previous tube or a 1:1000 dilution from the reaction vessel (beaker).
- 6. Plate aliquots of the  $10^{-2}$ ,  $10^{-3}$  and  $10^{-4}$  dilutions into duplicate petri plates.
- 7. Pour 15 20 mL of sterile molten TSA+ for bacteria into petri plates. Mix by rotating plates to disperse sample in the agar completely. Stack series of plates on trays and allow agar to solidify.
- 8. This completes run #1.
- 9. Repeat steps 1 through 7 for runs #2 and #3.
- 10. Incubate plates for 48 h +/- 2 h at 350 +/- 20 C. Count colonies and record as cfu/g.

# Blank:

- 1. Place beaker containing 100 g sterile DI water and magnetic stir bar in water bath (37° +/-2° C for solids, 25° +/-2° C for liquids.
- 2. Inoculate with 1.0 mL of test organism (approximately 108 CFU/mL).
- 3. At the longest designated time, transfer 0.1 mL of sample/bacteria mixture from beaker into a 9.9 mL D/E broth blank (10<sup>-2</sup> dilution). This is a 1:100 dilution.
- 4. Transfer 0.1 mL of the 10<sup>-2</sup> dilution mixture into a second 9.9 mL D/E broth blank (10<sup>-4</sup> dilution). This is a 1:100 dilution from the previous tube or a 1:10,000 dilution from the reaction vessel (beaker).
- 5. Plate aliquots of 10<sup>-4</sup> and 10<sup>-5</sup> dilutions in triplicate.
- 6. Pour 15 20 mL of sterile molten TSA+ for bacteria into petri plates. Mix by rotating plates to disperse sample in the agar completely. Stack series of plates on trays and allow agar to solidify.
- 7. Incubate plates for 48 h +/- 2 h at 350 +/- 20° C. Count colonies and record as cfu/g

# **Evaluation/Calculations:**

Formula for calculating percent reduction is given as follows:

% reduction = <u>blank count - sample count</u> X 100. blank count

This may also be reported as log<sub>10</sub> reduction.

### References:

ASTM Standard: E 1054-91, "Standard Practices for Evaluating Inactivators of Antimicrobial Agents Used in Disinfectant, Sanitizer, Antiseptic or Preserved Products."

AOAC Germicidal Spray Products and Disinfectants. <u>AOAC Official Methods of Analysis</u>, 16th Edition, (1995).

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Title: Time Kill
Effective Date: Date signed by
Sponsor

# **ATTACHMENT II**

**Test Material Identification** 





Title: Time Kill
Effective Date: Date signed by
Sponsor

# **Test Material Identification**

Sponsor Identification Number	Physical Description
3372-151	Liquid Soap
-	





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Title: Time Kill
Effective Date: Date signed by
Sponsor

ATTACHMENT III

Neutralizer Efficacy



# Title: Neutralizer Efficacy Effective Date: July 18, 2000

#### **NEUTRALIZER EFFICACY**

#### **PURPOSE**

This protocol is designed to determine the ability of physical (i.e., dilution, filtration, etc.) and/or chemical inactivators (i.e., Tween 80, Lecithin, etc.) to neutralize the killing action of antimicrobial agents found in disinfectants, sanitizers and antiseptics.

#### **JUSTIFICATION**

Whenever there is a need to assess the antimicrobial efficacy of a test material, the antimicrobial agent must be inactivated so that any viable survivors remaining after the antimicrobial has been applied, can reproduce sufficiently to produce detectable colonies. Inadequate neutralization results in potential overstatement of antimicrobial efficacy.

### REGULATORY AGENCY SUBMISSION

This study will be subject to regulatory agency (FDA) review.

# RECOMMENDED EQUIPMENT AND SUPPLIES

Reagents & Supplies

Candida albicans, ATCC 10231

E. coli, ATCC 8739

Pseudomonas aeruginosa, ATCC 9027

Staphylococcus aureus, ATCC 6538

Additional microorganisms included as appropriate

Trypticase soy agar (TSA)

Sabouraud dextrose agar (SDA)

Sterile Phosphate Buffered Saline (PBS)

Sterile Phosphate Buffered Saline with 0.05% Tween 80 (PBST)

0.1% peptone water (0.1% peptone and 0.9% NaCl) (PW)

Petri Dishes

Sterile centrifuge tubes

Sterile pipettes or pipette tips

Sterile polypropylene centrifuge tubes to serve as the sample container during the course of the study 38 x 200 and 25 x 150 mm screw-cap test tubes

Neutralizers as appropriate (i.e., polysorbates 80 & 20, lecithin, sodium thiosulfate, cysteine, thioglycolate, metabisulfite, magnesium sulfate, calcium carbonate, hydrochloric acid & sodium hydroxide [for pH adjustment, amines, sodium bisulfite, and others)

# Equipment

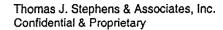
30-35°C and 20-25°C incubators

Various lab equipment including eppendorf pipettors, sterile tips, and vortex mixers Filtration equipment as appropriate

# **ASSAY PROCEDURE**

## **Inoculum Preparation**

Bacteria culture(s) will be transferred twice (once every 18-24 hours or as appropriate for a given test organism) and will be incubated, at a temperature and for a period, appropriate for the microorganism. The second transfer is made onto an agar plate or slant and the inoculum prepared by washing the plate or slant with 5-10ml of sterile PBS. Spore forming molds to be incubated until adequate sporulation is achieved.



Suspensions of all organisms will be poured into an appropriate size sterile tube and centrifuged at 4,000-5,000 rpm for approximately 10 minutes. The supernatant will be decanted, and the pellet resuspended by vortexing in 5-10 ml of PBS (PBST for mold).

Title: Neutralizer Efficacy

Effective Date: July 18, 2000

The concentration of test organisms will be adjusted spectrophotometrically in PBS (or PBST) to a concentration of approximately 1 x 10<sup>8</sup> cfu/ml. If no %T curve is available for a specific organism, that organism will be diluted 1:10 in PBS (PBST for molds) and used in the assay. Each adjusted organism(10<sup>8</sup>) will be serially diluted to approximately 10<sup>3</sup> - 10<sup>4</sup>CFU/ml; these are the inocula.

**Neutralizer Toxicity Test** 

One (1.0) ml of each test organism suspension (~10³) will be added to each of a series of tubes containing 9.0ml (the level of organism will now be approximately ~10² CFU/ml) of the diluent w/neutralizer added in typical range of concentrations and allowed to stand for approximately 30 minutes. At that time 1.0mL and 0.1mL aliquots will be plated in duplicate. Plates will be incubated, at a temperature and for a period, appropriate for the microorganism. The neutralizer will be considered non-toxic up to and including the concentration at which organism recovery is within +/- 50% of the inoculum control counts.

**Neutralizer Efficacy Test** 

One (1.0) ml of the approximately 10<sup>3</sup> CFU/ml organism suspension will be added to an 8.0 ml portion of neutralizing diluent. Finally, one (1.0) ml of test material (prepared as it is to be inoculated) will be added to this solution (the level of organism will now be approximately 10<sup>2</sup>- 10<sup>3</sup> CFU/ml). This mixture will be allowed to stand for approximately 30 minutes. At that time, a 0.1ml and a 1.0ml aliquot will be plated in duplicate. Plates will be incubated, at a temperature and for a period, appropriate for the microorganism. A viability test blank for this control will be run concurrently (see next section). The neutralizer will be considered effective if organism recovery is within +/- 50% of the counts for the neutralizer toxicity control. This neutralizer concentration is known as the Maximum Tolerated Concentration (MTC).

Inoculum Control

Each test organism will be serially diluted and plated to determine initial level of test organism (cfu/mL). Plate One mL of the suspension and 1.0 mL and 0.1mL from the 1:10 dilution (all in duplicate).

### PROTOCOL AMENDMENTS/DEVIATIONS

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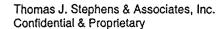
# **GOOD LABORATORY PRACTICE REGULATIONS**

This protocol will be conducted according to the principles of Good Laboratory Practice regulations. If it becomes necessary to change this approved protocol, verbal agreement to make such a change will be made between the study director and the Sponsor.

As soon as practical, the change and reasons for it will be written and signed by the study director and Sponsor. This document will then be attached to the protocol as an addendum. Examples of significant changes requiring Sponsor's approval are listed below:

- -Changes in the test materials to be tested
- -Changes in the concentrations of the test materials.
- -Changes in the assay procedure.





Title: Neutralizer Efficacy Effective Date: July 18, 2000

#### **FINAL REPORT**

A final report will be issued to the Sponsor which will include the following information:

- -Descriptions of the test material(s).
- -Dates of study initiation and completion
- -Any protocol deviation(s).
- -Study results, statistical calculations, and a summary/conclusion
- -A copy of this protocol

#### RETENTION OF TEST MATERIALS

At the completion of the study, remaining test materials will, at the Sponsor's expense, be returned to the Sponsor or destroyed at the Sponsor's expense.

# **NOTIFICATION OF CHEMICAL HAZARDS**

When sending test materials that are chemical hazards, the Sponsor must also supply appropriate MSDS information. Testing will not be done without this information. If hazardous materials are supplied, the materials will be returned to the Sponsor at the Sponsor's expense after completion of the study.

### MAINTENANCE OF RECORDS

When the final report is completed, all original records (including the study protocol, raw data, and any other records or forms used in the study) and a copy of the final report will be retained on file in the Stephens & Associates' archives for two years. When the archive time has expired, the study files are to either be sent to the Sponsor, archived at a different location at the Sponsor's expense, or destroyed. Any of these options may be carried out upon the Sponsor's request and notification.

# REFERENCES.

- 1. ASTM Standard, E1054-91
- Sutton, S. V. W. 1996. Neutralizer Evaluations as Control Experiments for Antimicrobail Effectiveness Tests. Chapter 3 in Handbook of Disinfectants and Antiseptics. Marcel-Dekker, New York.